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ACTION OF VARIOUS ANTICOAGULANTS ON HEMOLYMPHS OF LOBSTERS AND SPINY LOBSTERS

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Abstract

Effects of anticoagulants on hemolymph coagulation of lobsters are reported. Effects of some inhibitors on plasma clotting change in relation to molting stages of animals. Variations of electrophoretic and immunochemical protein patterns are analyzed. Complete coagulation inhibition shows that the plasma coagulogen exists as a mixture of soluble uncovalently crosslinked polymers. In lobsters the molecular weight of the lightest visible circulating unit is approximately 420,000 daltons. The weight interval between each of the four lighter polymers is about 200,000 daltons. Serine protease inhibitors impair plasma clotting and interact with cellular clumping.

Introduction

Several types of coagulation have been described in Arthropoda hemolymph (review in Grégoire, 1971). In Crustacea, three types of clotting processes have been defined (Tait and Gunn, 1918): cellular clumping (type A), complete plasma gelation (type C), and intermediates between these two types (type B).

In type A, the hemolymph remains fluid and only cellular factors seem to be involved in the clumping. This has not been studied in Crustacea, although Levin (1967) demonstrated that some cellular extracts from *Maia squinado* and *Carcinus maenas* clot on addition of endotoxin as cell lysates of *Limulus* do. In horseshoe crabs, the soluble coagulogen is contained within the hemocytes (Mürer *et al.*, 1975; Levin and Bang, 1968; Solum, 1970; Shishikura *et al.*, 1977). It is characterized by a small molecular weight and is converted into an insoluble coagulin by a serine protease enzyme localized in amoebocytes (Young *et al.*, 1972; Solum, 1973; Sullivan and Watson, 1975; Tai and Liu, 1977; Shishikura and Sekiguchi, 1978). The resulting clot is stabilized by noncovalent crosslinks (Tai *et al.*, 1977; Mosesson *et al.*, 1979).

In type C, the clottable factor ("fibrinogen") is a plasma protein. It has been studied in *Homarus* (Glavind, 1948; Duchateau and Florkin, 1954; Doolittle and Lorand, 1962; Stewart *et al.*, 1966), in *Panulirus interruptus* (Tyler and Scheer, 1945; Fuller and Doolittle, 1971a, b; Doolittle and Fuller, 1972) and in *Astacus leptodactylus* (Durliat and Vranckx, 1976). It has a high molecular weight and can be converted to a gel which seems to be crosslinked covalently by the action of a cellular transglutaminase (Bruner-Lorand *et al.*, 1966; Myhrman and Bruner-Lorand, 1970). Plasma factors of vertebrate blood have no effect on hemolymph gelation. Moreover, neither proteolysis nor fibrinolysis has been demonstrated in

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Abbreviations: NEM, N-ethyl maleimide; PCMB, parachloromercuribenzoate; PMSF, phenylmethane sulfonyl fluoride; DIFP, diisopropyl fluorophosphate; GME, glycine methyl ester; and DC, monodansylcadaverine.

Crustacea (Hawkey, 1970). These reports have led some authors to conclude that this plasma clotting has one step only: A soluble fibrinogen is transformed into an insoluble "fibrin-like" gel, (Fuller and Doolittle, 1971a, b) or more likely into a "fibrinogen-like" gel, by polymerization of a basic unit:

$$n \text{ fibrinogen} \xrightarrow[Ca^{2+}]{\text{transamidase}} \text{cross-linked (fibrinogen) n-gel (Lorand, 1972).}$$

In fact, a cellular clumping appears in each type of coagulation. A process similar to that in *Limulus* might exist in Crustacea either at the cellular level alone oı also at an early step in plasma clotting.

This study reports our attempts to prevent both plasma clotting and cellular clumping in lobster and spiny lobster hemolymphs by inhibitors of enzymes involved in the two types of clotting processes, and also to determine whether these two phenomena are interdependent, perhaps in a sequential manner.

Materials and Methods

Homarus vulgaris lobsters were a gift of "La Langouste" fisheries (Roscoff, France). Spiny lobsters (*Jasus lalendei, Jasus paulensis, Jasus frontalis, Panulirus regius, Palinurus vulgaris*) were obtained from local fish merchants. Molt stages were determined by examining uropods according to the method of Vranckx and Durliat (1978).

Serum and plasma were obtained from animals of both sexes in C3, C4, and premolt stages. Hemolymph was withdrawn from the periopod sinus and distributed into tubes. Sera were obtained according to a method previously described (Durliat and Vranckx, 1976). To delay or prevent coagulation, blood withdrawal was performed with a syringe containing a volume of chemicals identical to that of the hemolymph removed. Distilled water and sodium chloride solutions, at 1% or 3% (initial concentrations), were used to test the effects of dilution and osmolarity on clotting. Tests were also carried out in the presence of a 10% $CaCl_2$ solution. Three groups of anticoagulants, purchased from Sigma, were utilized in both hypotonic and isotonic media at the following initial concentrations:

1. As enzymatic inhibitors, we successively used: 10% sodium citrate or 5% EDTA solutions; 2.10^{-2} *M* NEM (N-ethyl-maleimide) with or without sodium citrate; 2.10^{-2} *M* PCMB (parachloromercuribenzoate); 2.10^{-2} *M* PMSF (phenylmethane sulfonyl fluoride) or DIFP (diisopropyl fluorophosphate) dissolved in 5% 2-propanol, or 10% soy bean trypsin inhibitor.

2. Among amines which inhibit transglutaminase-catalysed crosslinking of proteins, GME (Glycine methyl ester) (10^{-2} *M* or 10^{-1} *M*) and DC (monodansylcadaverine) (10^{-3} *M*), dissolved in 5% acetone, were tested.

3. As an inhibitor of membrane disruption, we used propanolol. It preserved the membrane of granules without preventing the disruption of hemocytes (Mürer *et al.*, 1975).

All experiments were performed at 4°C. Alteration of hemocytes was observed through an optical microscope. One aliquot of hemolymph was centrifuged immediately at 1000 rpm for 5 min and the plasma was separated from hemocytes. The other portion was left undisturbed. Each sample was examined as soon as possible and again after storage (one month at −30°C). Uncytolysed hemocytes quickly separated from plasma were treated with distilled water, HCl, and NaOH, as described by Solum (1973).

Protamine sulfate gelation tests were carried out as described by Gurewich and Hutchinson (1971).

Electrophoretic analyses were performed on polyacrylamide gradient gels (4–30% and 2–16%) in 0.15 *M* Tris-borate buffer at pH 8.2 for 24 h at 50 V, or at pH 9.6 with approximately 100 V for 48 h. A protein calibration kit (Pharmacia) was used to estimate molecular weights.

We injected rabbits with pooled whole hemolymphs (citrated plasma and cell lysates) from lobsters of both sexes in premolt stages and also with pools of hemolymphs from spiny lobsters corresponding to different molting stages, according to previously published procedures (Durliat and Vranckx, 1976). All samples were tested against homologous anti-hemolymph sera in Tris barbital buffer of pH 8.6, μ:0.03, as given in the reference. Occasionally, line immunoelectrophoresis was also performed by the method of Krøll (1973).

Results

Results of coagulation assays were nearly the same in lobsters and spiny lobsters, regardless of sex. Data are summarized in Table I.

In the absence of any anticoagulant, a cellular clump appeared. In freshly caught animals only, this clot then underwent a retraction. After a lapse of some minutes, the whole opaque hemolymph gelled and formed a firm transparent unretracted clot in the tube. In hypotonic medium, cellular lysis was very fast. Distilled water and 1% and 3% NaCl solutions had little effect on coagulation. Dilution slightly delayed clotting and produced a less firm gel. By contrast, with a 10% $CaCl_2$ solution, coagulation was even faster than in the reference sample.

Table I

Effects of anticoagulants on the hemolymph of Homarus vulgaris. *Clotting tests were performed on 20 lobsters. Intensity of responses is given by the following symbols: (−) represents no reaction; (+) is a weak reaction; (±) is a doubtful reaction; (++) is a moderate reaction, and (+++) is a strong reaction.*

Inhibitors \ Coagulation mechanisms	Cellular clump	Retraction of hemocyte clump	Plasma gelation
without	+++	+(+)	+++
distilled water	+++	++	++
NaCl 1%	++	+++	++
NaCl 3%	++	++	++
Sodium citrate, H_2O	+++	++	−
Sodium citrate, NaCl	++	−	−
NEM, H_2O	+(+)	−	−
NEM, NaCl	−	−	−
NEM, Sodium citrate, H_2O	+	−	−
NEM, Sodium citrate, NaCl	−	−	−
PSMF, H_2O	++	+	+
PSMF, NaCl	±	−	+
DIFP, H_2O	+	−	±
DIFP, NaCl	±	−	±
Trypsin inhibitor	±	−	−
NEM, PMSF, H_2O	+	−	−
NEM, PMSF, NaCl	−	−	−
Propanolol, NaCl	+	−	±
GME, H_2O	+++	+++	−
GME, NaCl	+++	+++	−
DC, H_2O	++	+	±
DC, NaCl	++	+	−

When blood samples were withdrawn with an anticoagulant in the isotonic medium, immediately centrifuged, and the cells discarded, no cellular phenomenon was observed and the plasma remained fluid.

When hemolymphs were not centrifuged, changes varied according to the inhibitor used. Sodium citrate and EDTA solutions did not inhibit cellular lysis but prevented plasma clotting. As NEM prevented cellular aggregation, the hemocytes sedimented but did not form networks or cellular clumps. This effect was more marked in the presence of Na and sodium citrate ions. The amoebocytes were preserved, and plasma did not coagulate. PMSF delayed gelling but did not inhibit plasma clotting, except in one animal in the C3 stage. Although the hemocytes were always quickly lysed, the clumping and its retraction were sometimes disturbed (in NaCl medium). Moreover, the coagulated plasma was soft and easily dissociated. DIFP and the trypsin inhibitor sometimes completely abolished or considerably delayed both plasma gelation and cellular clumping. Propanolol also delayed plasma gelation, which remained incomplete. The clotting time of *Homarus* plasma was considerably extended by amines that inhibit the crosslinking of vertebrate fibrin. GME and DC entirely inhibited plasma clotting but were without action on hemocytes, which formed a clot with a strong retraction of the coagulum.

The coagulated cell lysate was dissolved in 0.2 N HCl and then reprecipitated by neutralization with 0.2 N NaOH.

Principal data on electrophoretic analysis are presented in Tables II and III.

TABLE II

Effects of inhibitors on electrophoretical patterns of the hemolymph of Homarus vulgaris. *Experiments were performed on 10 lobsters. Supplementary bands are indicated by (a) fraction of about 420,000 daltons; (b) fraction with a molecular weight of approximately 200,000 daltons, which was only visible on gels running 48 h at pH 9.5 when the polymer scale was dissociated, and (c) aggregate with a high molecular weight. Symbols: (–), no reaction; (+), weak reaction; (±), doubtful reaction; (++), moderate reaction; (+++), strong reaction.*

Inhibitors \ Patterns	Polymer scale	Supplementary bands	Deposits on the top of gels	Plasmatic clotting
None	—	—		+++
Sodium citrate, H_2O	4–5 bands	a ±		–
Sodium citrate, NaCl				
NEM, H_2O	4–5 bands	—		–
NEM, NaCl				
NEM, Sodium citrate, H_2O	4–5 bands	a		–
NEM, Sodium citrate, NaCl		a		
NEM, Sodium citrate, NaCl centrifuged		b		
PMSF, H_2O	–	c	++	+
PMSF, NaCl	–	—		+++
PMSF, NaCl, centrifuged	–	b		+
DIFP, NaCl, centrifuged	–	b		++
NEM, PMSF, H_2O	5–6 bands	b	++	–
Propanolol, NaCl	–			++
Propanolol, NaCl centrifuged	7 bands			–
GME, 10^{-1} *M*	3–4 bands	a ± b		–
GME, 10^{-2} *M*, NaCl	8 bands	a + b		–
DC, H_2O	–	a + b		+
DC, H_2O, centrifuged	4–5 bands			–
DC, NaCl, centrifuged	7 bands	a + b		–

TABLE III

Effects of inhibitors on electrophoretical patterns of the Jasus lalendei *hemolymph. Experiments were carried out on 15 spiny lobsters. Additional bands are indicated by (a) fraction running in the area of 330,000 daltons; (b) fraction running slightly above the hemocyanin subunits in the area of 140,000 daltons; (c) aggregate with a high molecular weight, and (d) fraction running above 16 S hemocyanin. Other symbols: (—), no reaction; (+) weak reaction; (±), doubtful reaction; (++), moderate reaction; (+++), strong reaction.*

Patterns / Inhibitors	Polymer scale	Additional bands	Plasmatic clotting
None	—	—	+++
Sodium citrate	5–6 bands	—	–
NEM	5–6 bands	a + b	–
NEM + EDTA	6–7 bands	2–3b 2d	–
PMSF, H_2O	—	c	++
PMSF, Propanol	—	b c	±
Propanol	—	b c	+
DIFP	5–6 bands	b	–
Trypsin inhibitor	5–6 bands	c	–
GME, 0.01 *M*	7–8 bands	d	–
GME, 0.1 *M*	5–6 bands	b d	–
DC	5–6 bands	b c	–
Triton	—	—	+++

In standard conditions (gels 4–30%, pH 8.2 and migration for 24 h at 50 V), *Homarus vulgaris* hemolymphs in the presence of any anticoagulant that inhibited plasma gelation showed a soluble polymer scale in addition to the serum pattern (Figs. 1a, b, c). According to the inhibitor used, this scale of polymers exhibited a more or less large number of bands in the different plasmas. These fractions represented the plasma clottable factor, since fluorescence appeared with DC above the oligomer of hemocyanin and up to the most cathodic fraction of high molecular weight. The number of polymers was especially high with saline solutions of propanolol after centrifugation and with diluted amines, *i.e.* with products which did not entirely inhibit this gelation. When GME (which like DC acted competitively) was more concentrated, the number of fractions decreased without going below four. With inhibitors of the crosslinking phase (sodium citrate or NEM), the freshly centrifuged samples showed 4–5 bands. These different scales were much more evident in 2–16% gels (Fig. 1d). Moreover, in samples that contained sodium citrate, NEM with sodium citrate, GME, or DC, one supplementary fraction appeared above the hemocyanin oligomer. In samples with PMSF H_2O, one aggregate of important molecular weight was present and some fractions did not enter the gel.

The estimated weights of the different scale units were 420,000; 670,000; 850,000; 1,050,000; and 1,200,000 (fluorescence of DC conjugates used as reference to clottable products). The main hemocyanin fraction was estimated to be 385,000 daltons. With dilute GME or DC, the progression between each additional band of the polymer scale was about 100,000 daltons.

However, when the running time of electrophoresis was lengthened so that the proteins could reach the "pore limit" of the gel, the different fractions of these scales began to smear (Figs. 1e, f). All the polymer scales disappeared when the experiments were carried out for 48 h at 100 V, with a buffer at pH 9.5, except in samples with DC or GME. Moreover, with centrifuged samples obtained in the

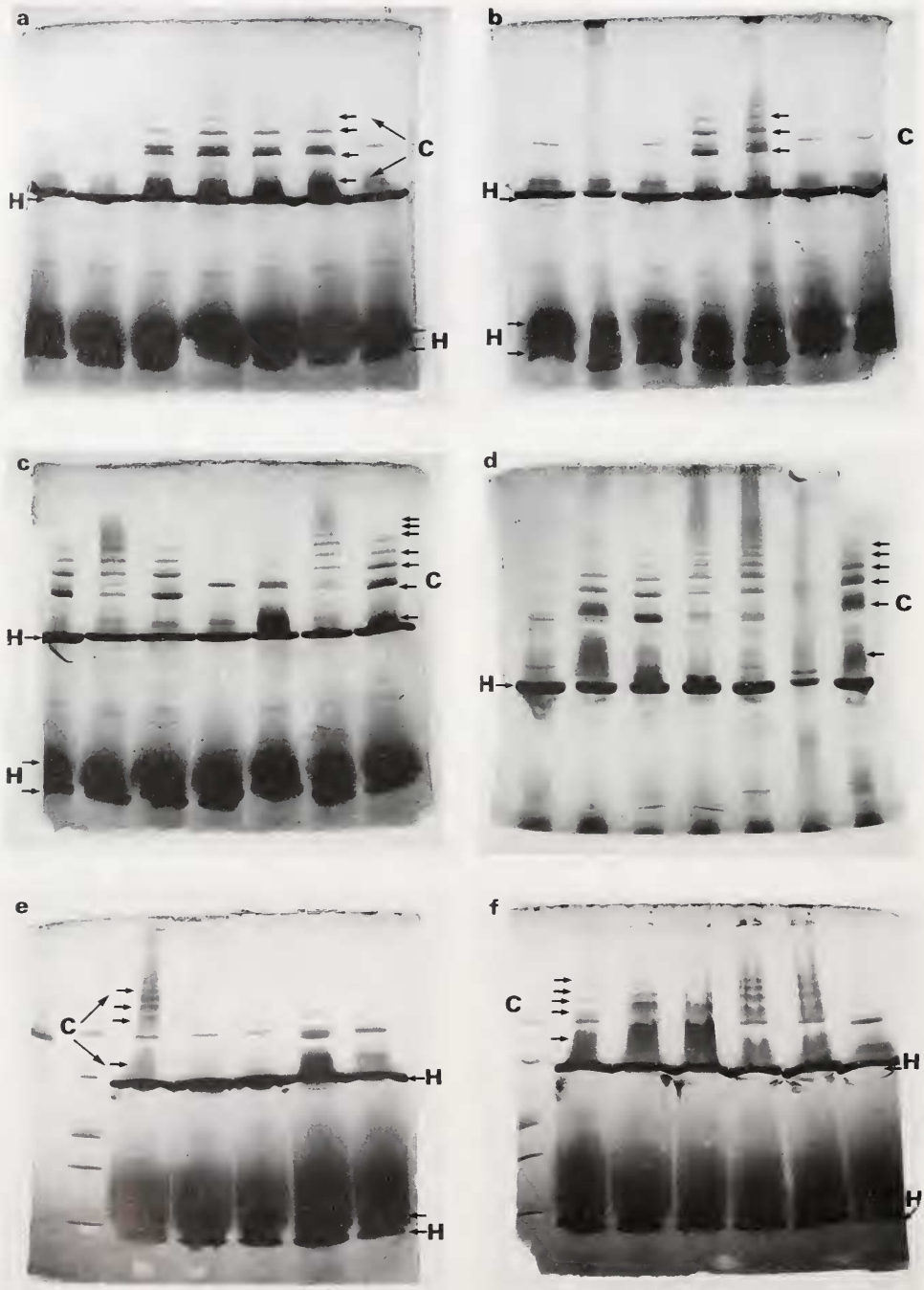

FIGURE 1. Effects of inhibitors on electrophoretic patterns of hemolymph from *Homarus vulgaris* on acrylamide gradient gels. (a), (b) and (c) show electrophoreses performed on 4–30% gels in standard conditions. (a) From left to right patterns of samples with NaCl 3%, propanolol, NEM sodium citrate H_2O, NEM sodium citrate NaCl, sodium citrate H_2O, sodium citrate NaCl, and serum. (b) From left

presence of saline solutions of NEM sodium citrate, PMSF, DC, or diluted GME, one band was more visible in the area of 200,000–250,000 daltons than with the other plasmas. Thus, fluorescence in the DC extract appeared in the scale of polymers but also in the area of 200,000–250,000 daltons as well as in that of 100,000 daltons.

When all blood samples, centrifuged or uncentrifuged, were submitted to electrophoresis after freezing storage, the number of polymers in the scale was higher in the uncentrifuged samples than in the centrifuged ones.

In spiny lobsters, electrophoresis was performed mainly on hemolymphs of *Jasus lalendei* and *Panulirus regius*. Data were similar to those reported in *Homarus* (Table III) but the plasma gel was firmer. One additional band was always present in the samples obtained with NEM, NEM in EDTA, or DC, and frequently with GME, PMSF (even after plasma clotting), or DIFP (Figs. 2a, b). This band ran slightly above the hemocyanin subunits, in the area of 140,000 daltons. This fraction was more easily visible when the hemocyanin oligomer was dissociated. The highest fraction of the polymer scale always appeared with PMSF or propanolol, but one intermediary band from this scale was absent; sometimes the same observations were noted either with a trypsin inhibitor or DC. Moreover, one or two additional fractions running between the hemocyanin hexamer and "fibrinogen" appeared in samples of GME or NEM in EDTA. In NEM one more easily visible fraction appeared in the area of 330,000 daltons (Figs. 2c, d).

In *Homarus vulgaris* a maximum of 13 precipitate lines was revealed by crossed immunoelectrophoresis (Fig. 3). Fractions present in all sera and plasmas were: 1 (hemocyanin), 3, 4, 8, and 10 (for identification of different fractions see Fig. 3). The presence or absence of other arcs depended on the inhibitors used, the experimental conditions, and on the sex and molting stages of the animals. All hemolymphs from female animals showed a group of cathodic precipitate lines (group 11), built up from two or three cross-reacting fractions, which did not appear in hemolymphs from male animals. In most experiments the thin cathodic fraction 12 was evident with whole hemolymphs but not with plasmas separated from uncytolysed amoebocytes. It was probably of cellular origin. Other proteins seemed to depend on the anticoagulant used. With DC a faint cathodic line 13 was shown and did not seem to relate to group 12, since it was present in the centrifuged sample. Two other fractions sometimes occurred both in sera and in plasmas: the very thin precipitate line 9, whose variations were not easily distinguishable, and the fraction 2, which moved into agarose as the hemocyanin did. These proteins may not have been connected with the clotting process, since they were absent in certain animals. When plasma gelation was inhibited, the soluble fibrinogen-like factor was always evidenced by a single precipitate line, 5 (Fig. 4), but the relative mobility of this factor 5 versus 4 varied according to the inhibitor used. Further-

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to right patterns of samples with PMSF NaCl, PMSF H_2O, centrifuged PMSF NaCl, NEM H_2O, NEM-PMSF H_2O, NaCl, and serum. (c) From left to right patterns of hemolymphs with DC, propanolol NaCl, NEM sodium citrate NaCl, H_2O, concentrated GME-NaCl, and GME H_2O. (d) Electrophoresis carried out on 2–16% gradient gels. From left to right patterns of serum and plasmas with sodium citrate, NEM sodium citrate, GME-NaCl, propanolol-NaCl, NEM-PMSF-H_2O, and centrifuged DC. (e) and (f) Electrophoreses performed on 4–30% gels with a high pH buffer. (e) From left to right: molecular weight standards, centrifuged plasmas with saline solutions of DC, DIFP, PMSF, NEM sodium citrate, and serum. (f) From left to right: molecular weight standards, plasmas with EDTA, concentrated GME after and without centrifugation, diluted GME after and without centrifugation, and serum. H shows the hemocyanin and C represents the different fractions of the clottable factor.

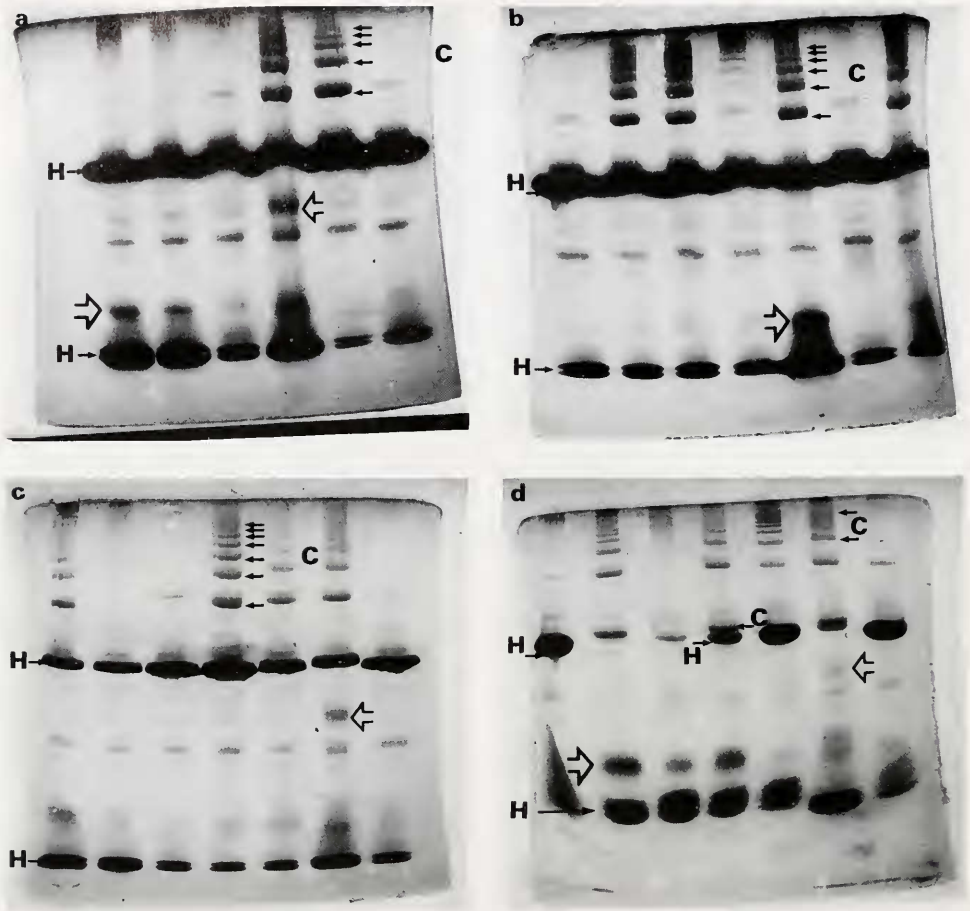

FIGURE 2. Effects of inhibitors on electrophoretical patterns of hemolymph from *Jasus lalendei* on acrylamide gradient gels (4–30%). (a) From left to right patterns of plasmas with propanolol, PMSF propanol, PMSF H_2O, NEM, sodium citrate, and serum. (b) From left to right patterns of serum, then samples obtained with concentrated and diluted GME, trypsin inhibitor, DC, triton, and NEM. (c) From left to right patterns of plasmas with DC, PMSF propanol, PMSF H_2O, diluted GME, concentrated GME, NEM, and serum. (d) From left to right patterns of plasmas with trypsin inhibitor, DC, PMSF propanol, GME, sodium citrate NEM, and serum. (a) and (b) Experiments were performed on animals in intermolt. (c) and (d) Experiments were carried out on animals in postmolt stage. The arrows show the additional fractions. H represents hemocyanin; C represents the different fractions of the clottable factor.

more, the fractions 6 and 7 seemed bound to the presence of this coagulogen but were not evident in each animal plasma. However, it must be noted that all these anticoagulant-dependent proteins, except 5, occurred in small amounts with faint precipitate lines, which were sometimes difficult to detect.

Analogous results were obtained in *Jasus lalendei*. Both crossed and line immunoelectrophoreses showed that some serine protease inhibitors acted upon the blood to prevent its gelation in some cases, since the clottable factor was evident in plasma with a trypsin inhibitor. Moreover, two fractions cross-reacting more or

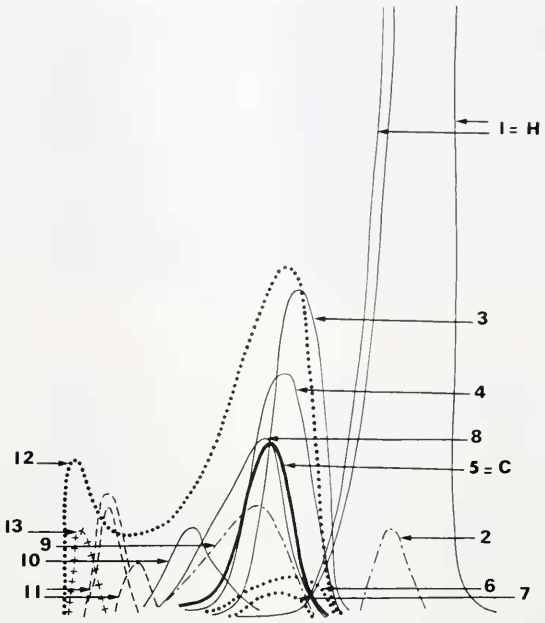

FIGURE 3. Schematic representation of crossed immunoelectrophoresis of the hemolymph from *Homarus vulgaris*. H shows the hemocyanin and C represents the clottable factor.

less completely with the fibrinogen of the NEM sample were observed in the PMSF H_2O hemolymph (Fig. 5).

DISCUSSION

In lobsters and spiny lobsters, hemolymph coagulation includes morphological changes in amoebocytes, followed by a release reaction, a cellular aggregation, and a beginning of retraction. This cellular transformation is rapidly hidden by the gelling of the whole plasma, which forms an "unretracted" clot. With inhibitors which delay whole-plasma clotting, but do not inhibit amoebocyte aggregation, complete retraction of the cellular clump is observed, recalling the thrombosthenin effect of vertebrate platelets. When plasma clotting is prevented, the "fibrinogen-like factor" always appears as a polymer scale in acrylamide gradient gels (minimum four bands), but as a single precipitate line in crossed immunoelectrophoresis. This electrophoretic heterogeneity is in agreement with the ultracentrifugation analysis of Duchateau and Florkin (1954) and Stewart *et al.* (1966), who have found both 21 S and 17–18 S components in the blood of *Homarus*. Fuller and Doolittle (1971a) have reported on the presence of 14.5 S and 19.4 S fibrinogen in *Panulirus interruptus*. Moreover, the latter authors have shown through disc electrophoresis that fibrinogen A is itself heterogeneous. With our specimens, DC situates these polymers from about 420,000 daltons (above 16 S hemocyanin) up to the top of the acrylamide gel. Fuller and Doolittle (1971a, b) and Doolittle and Fuller (1972), indicate the same weight for the "monomer" (fibrinogen B) of *Panulirus*. However, our "dimer" is not a multiple of 420,000, since the weight increase is of about 200,000 daltons between each of the four lighter bands. This could

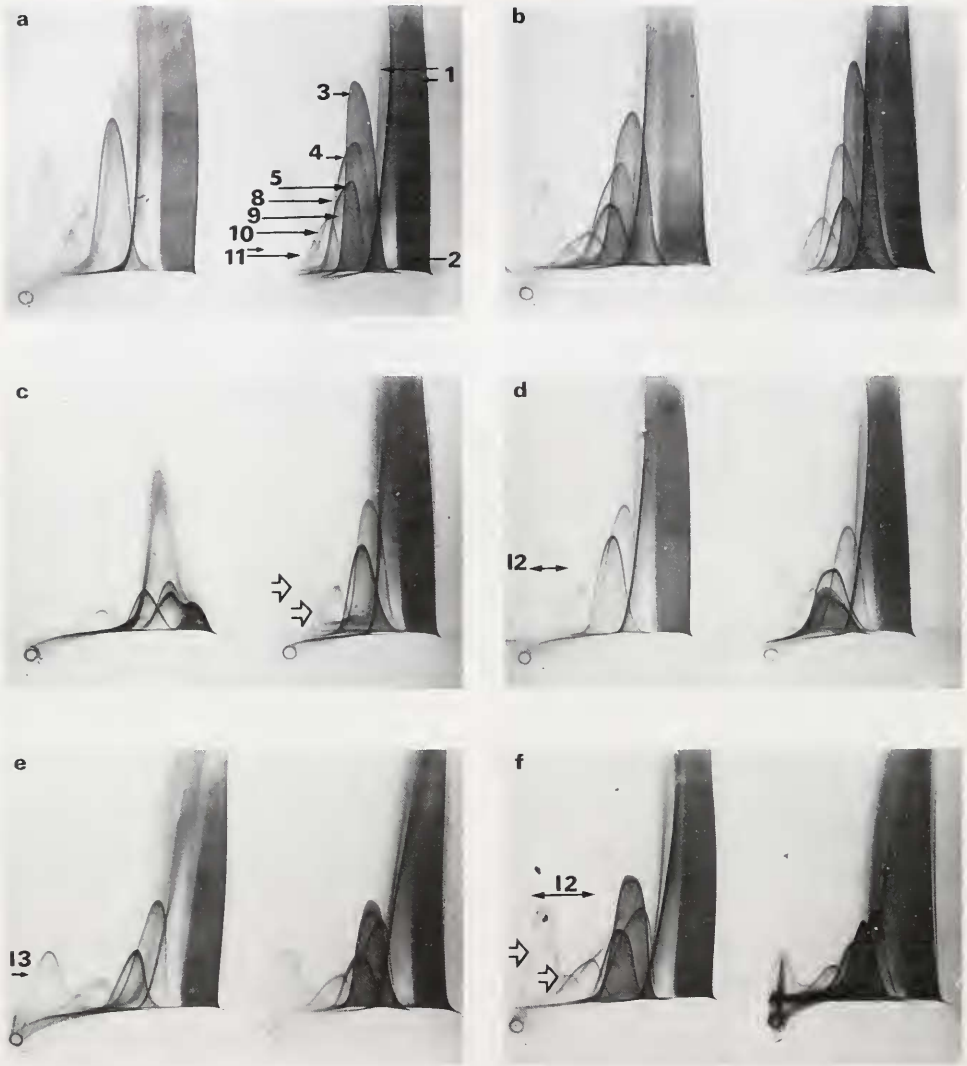

FIGURE 4. Crossed immunoelectrophoresis of hemolymphs from *Homarus vulgaris*. (a) Samples of serum and plasma with sodium citrate NaCl. (b) Plasmas with sodium citrate H_2O and NEM sodium citrate. (c) Samples with PMSF H_2O (dissociated after dialysis against a stripping buffer) and PMSF NaCl after centrifugation. (d) Plasmas with propanolol without and after centrifugation. (e) Plasmas with DC without and after centrifugation. (f) Samples with GME without and after centrifugation. The white arrow shows cross-reacting fractions of group 11. Each sample of serum and plasma contains 100 μg of proteins—An agarose gel containing 4 μl/cm^2 of lobster antiplasma serum is used.

perhaps better explain the shift from 18 S to 21 S evidenced through ultracentrifugation in *Homarus*. The pore gradient gel gives a true estimation of the molecular weights, since Fuller *et al.* (1972) have shown by electron microscopy that the molecule of "fibrinogen" of *Panulirus interruptus*, at least for the monomer, is spheroid.

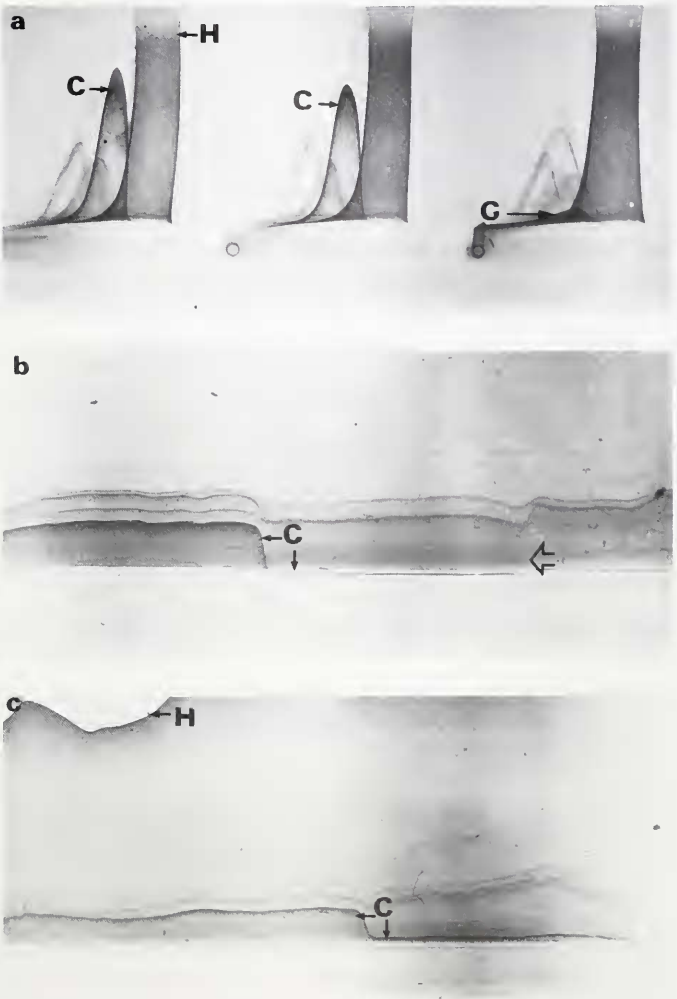

FIGURE 5. Immunoelectrophoreses of hemolymphs from *Jasus lalendei*. An agarose gel containing 15 μl/cm^2 of spiny lobster antiplasma serum is used. (a) Crossed immunoelectrophoresis: from left to right plasmas with concentrated GME, diluted GME, and trypsin inhibitor; each antigen contains 300 μg of plasma proteins. (b) and (c) Line immunoelectrophoreses: (b) From left to right plasmas with NEM, PMSF H_2O, and PMSF propanol. (c) From left to right samples of plasmas with GME and trypsin inhibitor. Antigens contain 600 μg of proteins in each line. H shows the hemocyanin and C represents the clottable factor. The arrow shows a fraction cross-reacting with the fibrinogen-like factor.

In supernatants separated from intact hemocytes (with saline solutions of NEM sodium citrate, PMSF, GME, or DC), one additional fraction appears when experiments are carried out for 48 h with a buffer at pH 9.5. In *Homarus* this fraction has approximately 240,000 daltons. Since the fluorescence of DC conjugates then is partly located at this place, it is possible that this component represents either the true monomer of "fibrinogen" or a subunit of soluble fibrin. This result is in agreement with the earliest reports of Fuller and Doolittle (1971), who have shown that the clottable factor is built up from two subunits of about 200,000 daltons.

However this band is not found in plasmas of *Jasus lalendei* and *Panulirus regius* (except in NEM solutions) although the polymer scale shows the same pattern as in *Homarus*.

With amines the clotting time of plasmas of *Homarus vulgaris* and spiny lobsters is considerably prolonged by the inhibition of fibrin crosslinkings, as reported by Lorand *et al.* (1963, 1964, 1965) in *Homarus americanus*. When low doses of GME or DC are used, the higher number of bands visible in the polymer scale leads us to think that the coagulation is initiated.

Indeed, this scale might not be the visualization of a biological phenomenon, but only the result of the experimental conditions since hemocyanin from *Homarus*, observed mainly as 24 S in ultracentrifugation, appears chiefly as 16 S and 5 S in our electrophoreses. In spiny lobsters, one supplementary band occurs in some samples (Table III), especially when 16 S is dissociated. It is probably a stable dimer (7 S) of subunits of hemocyanin. Dissociations of the native 24 S and 16 S hemocyanins during electrophoresis could be not only related to pH changes, but also connected with the ionic strength of the buffer and with the control of its temperature (Busselen, 1970). Thus, the ratio between the low ionic strength of the buffer and higher strength of the internal medium of sea animals could explain why we never observe 24 S in electrophoreses of the *Homarus* hemolymph. However, some other marine Crustacea (*Maia squinado, Cancer pagurus*) show 24 S hemocyanin in gradient gel electrophoresis. With a buffer system able to dissociate oligomeric proteins (hemocyanin and perhaps the fibrinogen-like factor) more or less completely during electrophoresis, the pattern of whole hemolymphs can vary according to migration time. Freezing promotes polymerization of coagulogen, sometimes to the point of complete clotting, for instance, with samples in DC after several freeze-thaw cycles. Since "fibrinogen" shows several peaks in ultracentrifugation, the polymer scale does not seem due to artifacts. These polymers are converted into insoluble fibrin by a transglutaminase enzyme, as in vertebrate clotting. However, no particular fraction related to cellular transamidase has been identified, perhaps due to the small amount of this protein. In crossed immunoelectrophoresis, changes in some fractions have been noted according to the sample studied, but their variations do not seem related to this enzyme. Thus, the fractions of group 11 observed only in female lobsters and spiny lobsters very likely represent vitellogenesis-related proteins, and fraction 2 could be dependent on the molting stage.

The macruran coagulation process seems more complex than previously claimed, since different inhibitors act upon the several steps of this plasma clotting. With serine protease inhibitors, plasma clotting is either totally absent (sometimes using DIFP, the trypsin inhibitor or, as in Joubert, 1954, the phosphate buffer) or delayed and abnormal. The soft gel, when formed, is easily dissociated mechanically, probably due to some clotting defects. However, results vary according to the animals investigated, perhaps in relation to their molting stages. Indeed, with the trypsin inhibitor, when plasma clotting is prevented in *Jasus lalendei*, the bands of the polymer scale are present, as with other anticoagulants, but are finer and slightly shifted towards higher molecular weights (Fig. 2b). A small proteolysis could occur on the clottable factor, as it does on the cellular coagulogen of *Tachypleus* (Nakamura *et al.*, 1976) and *Limulus* (Tai *et al.*, 1977), which was at first found not to be enzymatically cleaved (Solum, 1970). Thus, in presence of the soy bean trypsin inhibitor, these polymers might perhaps represent aggregates of "fibrinogen," even though the other polymer scales could be the visualization of uncrosslinked polymers of fibrin. It is exciting to think that the fibrinogen might be a

short-lived transitory step, and that the circulating state could be soluble fibrin complexes. The protamine sulfate gelation test, which precipitates the soluble complexes of fibrin from vertebrates (Gurewich and Hutchinson, 1971), does not give similar results in these Decapoda. Nevertheless, whether the circulating state is fibrin or fibrinogen, this clottable factor should be called coagulin and its precursor coagulogen, as defined by Needham (1970), and more precisely plasmatic coagulogen and coagulin in order to differentiate them from cellular coagulogen and coagulin of *Limulus*.

These inhibitors also act at the cellular level, since they more or less prevent the clumping of hemocytes. It seems that some factors of coagulation are located within the amoebocytes and perhaps within the granules, since propanolol retards the plasma gelling. Analogous facts are reported on *Limulus* (Mürer *et al.*, 1975). Cell lysates from lobsters form a gel, which behaves as do coagulated cell lysates of *Limulus* (Solum, 1973). Cell lysates of *Homarus* seem to contain a coagulogen and an enzymatic system able to convert this coagulogen into a gel, which includes the aggregate cellular mass. This cellular clot, which is considerably delayed by inhibitors affecting serine hydroxyl and sulfhydryl groups, has been found in all Decapoda investigated (*Maia squinado, Cancer pagurus, Carcinus maenas, Xantho, Macropipus puber, Astacus leptodactylus, Homarus vulgaris, Nephrops norvegicus* and different species of spiny lobsters), whether or not they exhibit plasma clotting. This reaction is more or less strong and rapid according to the species studied (important in *Maia, Cancer*, and *Limulus polyphemus*) and is related to the molt stage and to the physiological state of the subjects (unpublished results).

In conclusion, a cellular coagulation analogous to that of the *Limulus* has been shown in the Decapoda studied. Although thrombin (a serine protease) does not promote plasma clotting of lobster hemolymphs, our results suggest that a thrombin-like enzyme, in addition to transglutaminase, may be involved in this process. Macruran plasmatic clotting seems to depend on a multienzymatic system. These enzymes could perhaps act in cascade-like sequences, as in some vertebrate coagulation mechanisms.

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